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Comparison of the Sequences of the Internal Transcribed Spacer Regions and *PbGP43* Genes of *Paracoccidioides brasiliensis* from Patients and Armadillos (*Dasypus novemcinctus*)

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***Paracoccidioides brasiliensis* isolates from 10 nine-banded armadillos (*Dasypus novemcinctus*) were comparable with 19 clinical isolates by sequence analysis of the *PbGP43* gene and ribosomal internal transcribed spacer 1 (ITS1) and ITS2 and by random amplified polymorphic DNA. In this original ITS study, eight isolates differed by one or three sites among five total substitution sites.**

In humans, the thermally dimorphic fungus *Paracoccidioides brasiliensis* causes paracoccidioidomycosis (PCM), a systemic granulomatous mycosis prevalent in rural areas of Latin American countries. Infection generally occurs by inhalation of conidia, which transform into pathogenic yeasts in the pulmonary alveoli (18). Nine-banded armadillos (*Dasypus novemcinctus*) have recently been considered a natural reservoir of *P. brasiliensis* (2, 9, 21, 24, 29). Apparently, different organs of individual armadillos can be infected with *P. brasiliensis* bearing distinct genotypes and virulence capacities, but the data on genetic polymorphism have so far been restricted to samples isolated from a few animals (25, 26).

Recently, *P. brasiliensis* strains with typical morphology have been isolated from the spleen, liver, and mesenteric lymph nodes of 10 armadillos captured in the counties of Botucatu, Pratânia, and Manduri (1, 11) (Fig. 1), located in the area of Botucatu, São Paulo state, Brazil, where PCM is endemic (17). We have shown that these isolates are able to cause PCM infection of various degrees in hamsters (11).

These samples have been compared at the DNA sequencing level with clinical isolates Bt60, Bt84, Bt85 (from the same area as shown in Fig. 1), Pb265, and Pb1 to Pb16 (detailed in reference 19) (Pb16 was isolated from soil). Our aim was to distinguish between human and armadillo isolates based on the polymorphism of two loci: the internal transcribed spacer 1 (ITS1) and ITS2 of the ribosomal DNA complex and the *PbGP43* gene (8), both already used in the identification of *P. brasiliensis* by PCR (4, 10, 12, 27, 28). *PbGP43* (1,329-bp long, with one 78-bp intron) encodes the major gp43 fungal antigen (8, 23, 30), and its polymorphism has been previously characterized by using Pb1 to Pb16 (19). The ITS region has been successfully used for typing of pathogenic fungi (13), including *Histoplasma capsulatum* (14), which is genetically related to *P. brasiliensis*, as inferred from 18S and ITS analysis (3, 22). Our interest was to verify its usefulness in *P. brasiliensis* intraspecific differentiation.

Fungal isolates were maintained as yeasts at 35°C (11, 19); DNA extraction was carried out by using a glass beads protocol (31) or as previously described (7, 19) for Pb1 to Pb16. *PbGP43* exon 2 was PCR-amplified according to standard protocols with specific primers 5′-TCATCTCACGTCGCATCTCACAT T-3′ (sense) and 5′-GGCTCCTCAAAGTCTGCCATGAGG AAG-3′ (antisense), which extend from nucleotide 733 to nucleotide 1,213 (8). Universal primers ITS4 (5′-TCCTCCGCT TATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCG TAACAAGG-3′) were used to generate a 634-bp PCR product that included the ITS1, 5.8S, and ITS2. The fragments were purified through MicroSpin S-400 HR columns (Amersham Pharmacia) and used as templates in sequencing reactions (DYEnamic Terminator Cycle Sequencing kit; Amersham Pharmacia). Both strands were sequenced in an ABI model 373A automated sequencer. For Pb1 to Pb16, the procedures for DNA amplification and sequencing of cloned fragments are detailed elsewhere (19).

A previous study (19) defined the existence of six *PbGP43* genotypes based on the distribution of 21 substitution sites, which occurred mostly in exon 2 (nucleotides 578 to 1166), generating predominantly nonsynonymous amino acid changes. The partial *PbGP43* sequences obtained in this work matched three of these genotypes, and most of them belonged in groups E and F (Table 1). Lymph node (LN-numbered) isolates seemed to fit preferentially in group E, while those from spleen (B-numbered) and liver (F-numbered) were mostly in group F. In a previous study (25), *PbGP43* sequences of three isolates from organs of the same armadillo were similar to those of either group F (spleen) or B (liver and lymph node).

We found two polymorphic sites in ITS1 and three in ITS2 (Table 2), but 73% of the sequences were identical to the consensus, which matched that previously deposited in GenBank (accession no. AF38360). The 5.8S subunit was con-

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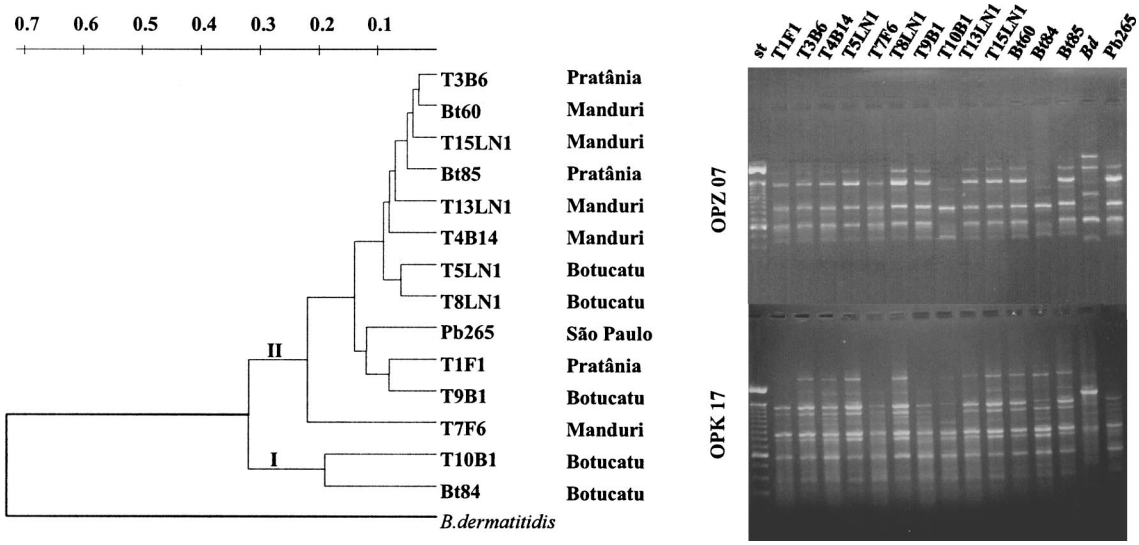

FIG. 1. (Left) dendrogram of *P. brasiliensis* isolated from humans (Bt60, Bt84, Bt85, and Pb265) and armadillos (T-named), including T1F1, T3B6, and T4B14 studied before (26), distributed according to a matrix of genetic similarity generated from the analysis of RAPD profiles with 24 primers. The data were assembled by using the neighbor-joining method, with *B. dermatitidis* (ATCC 26199) as an outgroup, which shared 27% genetic similarity. Main clades I and II and the county of origin are indicated. (Right) representative RAPD profiles (with primers OPZ07 and OPK17). st, 100-pb ladder (strongest band, 600 bp). The distance between the armadillos' burrows has been mapped by using the global positioning system (1). The approximate average distances were 33 km (Botucatu and Pratânia), 86 km (Botucatu and Manduri), and 62 km (Manduri and Pratânia). Animals from the same county lived between 0.35 and 9.88 km apart.

served. Site 518 (A to G) was mutated in five isolates and could be defining two *P. brasiliensis* genetic groups. However, a maximum-likelihood phylogenetic tree had weak bootstrap support and is not presented. Note that the ITS primer pair previously suggested for amplification of a fragment specific for *P. brasiliensis* (12) does not contain any polymorphic sites.

The *PbGP43* sequences of T10B1 and Bt84 were similar to that of Pb4, which together with those of Pb2 and Pb3 were phylogenetically distant from the others in a maximum-likelihood tree (19). These sequences are highly polymorphic and translate peculiarly basic gp43 isoforms, which contain some differential antibody epitopes (19, 20). Among the five isolates presently known to encode basic gp43 (Table 1), four (Bt84/Pb2, Pb4/T10B1) had polymorphic ITS of two different patterns (Table 2). Moreover, T10B1 and Bt84, both from Botucatu, shared the same clade I in a dendrogram resulting from random amplified polymorphic DNA (RAPD) analysis (Fig. 1). T10B1 was significantly more aggressive than the other armadillo isolates in the hamster experimental model, killing the animals after 2 weeks of intratesticular infection (11).

The biggest clade II of the RAPD tree assembled most of the armadillo isolates tested (Fig. 1), which did not carry any mutation in ITS (Table 1) and had similar *PbGP43* genotypes (Table 2). Our RAPD analysis, carried out as previously described (6) in a thermocycler (MJ Research, Inc.,Waltham, Mass.) with 24 random primers (Operon Technology), originally aimed at distinguishing the isolates geographically, as previously reported for *P. brasiliensis* (6). Indeed, their distribution into branches seemed to correlate with the county of origin.

In this communication, we showed the first genetic analysis of *P. brasiliensis* from a large number of armadillos and confirmed their similarity with clinical isolates by DNA sequencing. We showed the first sequence comparison of the ITS1 and ITS2 regions from many isolates, among which eight differed by one or three sites among five total substitution sites. Our

TABLE 1. *P. brasiliensis* isolates grouped according to nucleotide substitution sites in a consensus *PbGP43* sequence[a]

Group	*PbGP43* substitution sites[b]	Isolate(s) from indicated study	
		Previous[c]	Present
A	268, 578, 617, 628, **751, 763, 799, 830, 856, 872, 981, 1082, 1086, 1157, 1166**	Pb2, Pb3, Pb4	T10F1, Bt84
B	617, **799, 821, 852**	Pb15, Pb16	None
C	617, **799**	Pb1	None
D	589	Pb5, Pb10, Pb11	None
E	**874, 965**	Pb6, Pb7, Pb8, Pb14	T5LN1, T9B1, T13LN1, T15LN1
F	**874, 965, 1143**	Pb9, Pb12, Pb13	T1F1, T3B6, T4B14, T7F6, T8LN1, Bt60

[a] The *PbGP43* sequence was reported by Morais et al. (19). The sequences obtained in the present work covered only the sites shown in bold (part of exon 2). See reference 19 for details about the nucleotide substitutions, corresponding amino acid changes, and extensive discussion about the gp43 isoforms.
[b] The nucleotide numbers are the same as those used by Cisalpino et al. (8).
[c] The GenBank accession numbers for the isolates from the previous study are U26160 and AY00545 to AY005437.

TABLE 2. Polymorphic nucleotides found in the ribosomal ITS1 and ITS2 sequences from 30 *P. brasiliensis* isolates[a]

Site[b]	Consensus[c]	Isolate							
		Pb10	Pb8	Pb12	Pb13	Pb4	T10B1	Bt84	Pb2
113	G					A	A		
156	G							T	T
384	C					T	T		
518	A		G	G	G	G	G		
554	T	C							

[a] Twenty-two sequences corresponded to the consensus. Sequence polymorphisms were analyzed using the Megalign program of the Lasergene System (DNAstar Inc.).

[b] Site 1 corresponds to the first base of ITS1.

[c] The isolates with sequence consensus (same as GenBank accession no. AF38360) were Pb1, Pb3, Pb5, Pb6, Pb7, Pb9, Pb11, Pb14, Pb15, Pb16, T1F1, T3B6, T4B14, T5LN1, T7F6, T8LN1, T9B1, T13LN1, T15LN1, Bt60, Bt85, and Pb265.

results suggest the existence of two genetic groups, since those defined by *PbGP43* and RAPD analyses did not necessarily coincide with the ITS groups. We believe that both *PbGP43* and ITS will be useful in further genetic studies of *P. brasiliensis* similar to those that revealed intraspecific genetic groups and cryptic sex in *Coccidioides immitis* and *H. capsulatum* (5, 15, 16).

Nucleotide sequence accession numbers. The GenBank accession numbers for the new ITS sequences are AY374336 to AY374339.

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